USDA Technical Working Group Report on Honey Bee Toxicity Testing
July 8 and 9, 2009

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BACKGROUND

On July 8 – 9, 2009, the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) and Agricultural Research Service (ARS) sponsored, and the Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) hosted, a panel of expert scientists to address study designs for testing honey bee toxicity. The panel discussed the current status of (EPA and European) test protocols and identified potential areas for test refinement and expansion, seeking to create more standardized testing to increase consistency and develop a more uniform understanding of the study results.

Honey bee toxicity testing is among the key topic areas identified in the USDA Colony Collapse Disorder (CCD) Action Plan (http://www.ars.usda.gov/is/br/ccd/ccd_actionplan.pdf). Specifically, topic 3 of the Action Plan is to conduct research for identifying factors affecting honey bee health; the first goal among this topic is to *test the lethal and sub-lethal effects of neonicotinic [insecticides] and other pesticides used for crop protection*.

Introductions by Dr. Steven Bradbury, Director of the EPA Office of Pesticide Programs (OPP), and Dr. Donald Brady, Director of the Environmental Fate and Effects Division (EFED) of OPP, emphasized that the foundation of EPA's pesticide regulatory process is to use sound science for decision making and to advance the science of risk assessment in a transparent manner. Both speakers acknowledged the fruitful relationship between EPA and USDA, particularly as it relates to determining the potential effects of pesticides on pollinators.

The panel consisted of 12 presentations by speakers from U.S., French, and Canadian Government, University, and contract scientists, covering the following topics: ecological effects assessment, larval acute toxicity testing, adult acute toxicity testing, and chronic effects testing. Summaries of presentations and the panel's analysis of each topic appear below.

ECOLOGICAL EFFECTS ASSESSMENT

Part 1: Presentation Summaries

Overview of Current Ecological Risk Assessment—U.S.-EPA Perspective
Allen Vaughan: Senior Biologist, EFED, OPP, EPA

The terms and conditions for pesticide registration are determined by the toxicological profile shown by the data generated for each compound. When evaluating potential risks of pesticides, EPA evaluates single active ingredients in chemicals. The focus of the USDA-sponsored *Apis*-toxicity meeting is, therefore, directed at single compounds.
EPA currently relies on a tiered process of data collection. In determining registration and reregistration of outdoor use pesticides, data on terrestrial invertebrates (including but not limited to pollinating insects) currently required by EPA include, at minimum, honey bee acute toxicity data from acute contact (Tier 1) as defined in Title 40 of the Code of Federal Regulations[1]. Depending on the results of Tier 1 studies, EPA may also require data from foliar contact (Tier 2) and field toxicity (Tier 3) studies.
Specifically, if the study results in a contact LD_{50}<11µg/bee or greater, then a toxicity of residues on foliage study is required (Tier 2). If a pesticide is demonstrated to have prolonged toxicity of residues, its acute contact is greater than LD_{50}<11µg/bee, and/or if there are open literature studies indicating potential adverse effects to honey bee colonies, then a field pollinator study may be required (Tier 3). There are relatively detailed guidelines for Tier 1 (Guideline 850.3020)[2] and Tier 2 (Guideline 850.3030)[3] studies.

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[1] Code of Federal Regulations. 2010. Title 40. Protection of the Environment. Part 158. Data Requirements for Pesticides. Subpart G. Ecological Effects. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=3e03f428688048706ece042af826e4dc&rgn=div8&view=text&node=40:23.0.1.1.9.7.1.1&idno=40
[2] USEPA 1996. Ecological Effects Test Guidelines. OPPTS 850.3020. Honey Bee Acute Contact Toxicity. Office of Chemical Safety and Pollution Prevention (formerly Prevention, Pesticides and Toxic Substances_ EPA 712-C96-147. http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3020.pdf
[3] USEPA. 1996. Ecological Effects Test Guidelines. OPPTS 850.3030. Honey Bee Toxicity of Residues on Foliage. Office of Chemical Safety and Pollution Prevention (formerly Prevention, Pesticides and Toxic Substances_ EPA 712-C96-148.

However, issues of consistency (*i.e*., study design and methodology) often confound efforts to interpret these data in a regulatory context.– Although study guidelines exist for a field pollinator toxicity test (Guideline 850.3040)[4], actual study designs vary considerably, as these studies are typically hypothesis driven. These studies are typically required on a case by case basis, and EPA review and concurrence of a protocol is encouraged before the study is conducted. To satisfy the data requirements for pesticide registration, technical registrants either conduct the required studies, hire a contract laboratory to conduct the studies, or submit open literature and a rationale for why the open literature fulfills the data requirement.

With regard to honey bees, current methodology is suited to assess potential risk (exposure and hazard) from "contact" compounds (*i.e*., pesticide compounds that exhibit direct contact toxicity) in young adult forage bees. Current exposure and hazard tests are not designed to assess the toxicity of systemic compounds (such as neonicotinoids or ketoenols) that are taken up by the plant and distributed internally to plant tissues and may also persist longer in the environment; these chemistries present new pathways for exposure (*e.g*., ingestion of residues through pollen, nectar, and drinking water) in comparison to conventional contact-based pesticides, representing a significant need. Exposure pathways vary, and there is uncertainty regarding exposure test methodology for systemic pesticides as they are translocated to pollen or nectar.

Another deficiency of the current testing framework is the lack of testing protocols that measure a broad range of sublethal effects. The range of sublethal measurement endpoints for honey bee studies have not been thoroughly vetted, and in order to be useful for risk assessment purposes, measurement endpoints must be linked to regulatory risk assessment endpoints. Current regulatory assessment endpoints are those that result in effects to an organisms growth, reproduction and/or survival and are known to result in population (colony level) effects.

Data Needs:

- *Acute Oral Toxicity Testing*: Currently, EPA does not require this on a regular basis, but it is routinely required by the European Union, and so a vetted valid protocol currently exists.
- *Exposure Testing (Nectar and Pollen)*: Current testing tools are not adequate for assessing exposure to systemic compounds that may translocate to pollen and nectar.
- *Effects to Non-adult Bees*: Current Tier 1 and Tier 2 studies focus on adult forager bees. Potential effects on larvae, including sublethal effects, are not assessed in lower-tier testing.
- *Measurement and Assessment Endpoints*: Current study guidelines indicate that sublethal effect data are to be recorded; however, the definition and standardization of sublethal effect measurement endpoints have not been properly vetted.

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http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3030.pdf

[4] USEPA. 1996. Ecological Effects Test Guidelines. OPPTS 850.3040.Field Testing for Pollinators. Office of Chemical Safety and Pollution Prevention (formerly Prevention, Pesticides and Toxic Substances_ EPA 712-C96-148.
http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3040.pdf

- *Higher Tiered (Field Study) Testing Designs*: Standardized methods for these test have not been developed and are important to measure effects at the colony level under settings [closer] to actual use conditions.

Suggestions:

- Use EU protocol for oral acute and sublethal toxicity testing.
- Degradates and by-products should be identified and tested for effects.

EU Risk Assessment Overview and Data Requirements
Anne Alix: Agency Francaise de Securite Sanitaire Des Aliments (AFSSA)

The European Union (EU) requires an ecological risk assessment, for pesticides prior to the placing of these products on the market. This process includes an assessment of risks to honey bees. Risk to honey bees from exposure to pesticides is assessed according to the European and Mediterranean Plant Protection Organization (EPPO) standards PP 3/10 (2) (OEPP/EPPO) and includes a tiered progression of testing described by the guideline No. 170 (OECA/EPPO). Annexes II and III of Directive 91/414/EEC list respectively the data requirements aiming at characterizing potential effects of pesticides on arthropod species and the corresponding general risk assessment principles Decision-making criteria related to honey bees are provided in Annex VI. In addition to the regulatory text, guidance documents have been developed with the aim to provide harmonized test guidelines and risk assessment principles.

To be approved, pesticide formulations must demonstrate effectiveness for intended purpose, no harm posed to humans, and no adverse effects on environment.The conditions for applying pesticides for which the exposure of bees cannot be excluded are outlined in Directive 91/414/EEC and address all outdoor uses either through sprayng plants, or through soil or seed treatment. For spray treatments, bees are most likely exposed during the flowering period while they are foraging. Exposure is based on the application rate, as expressed in grams of the active ingredient/ha .

A risk assessment of sprayed treatments to honey bees may be performed through the calculation of a hazard quotient (HQ) which is the ratio between the exposure assessed by the application rate (expressed in gram ai/ha) and the toxicity (LD50 from acute contact and or oral exposure) of the active substance or the preparation. An HQ value of 50 and above suggests a potential risk of the use(s) to which correspond the application rate for honey bees, that calls for further considerations through a refined risk assessment as described below.

Screening level assessment for systemic compounds may focus on acute oral risks posed by exposure through residues in nectar and pollen. In these cases, the HQ is replaced by calculation of a Toxicity Exposure Ratio (TER). The TER (PED/PNEC) is the ratio of the Predicted Exposure Concentration (data on exposure through the nectar or pollen) to the Predicted No Effect Concentration (data from acute sub-acute or chronic toxicity testing).

Effects assessments involve studies performed on individual bees in the laboratory on in micro-colonies, or to whole colonies under semi-field or field conditions. Since intrinsic toxicity is not effectively predicted from testing adults alone, a reliable screening of toxic effects on honey bee larvae may be performed based on the method proposed by Oomen *et al.* (1992) and Aupinel *et al* (2005)utilizing micro colonies exposed through a spiked feeding solution and effects on the brood development to determine a NOEL. Dosage is based on the maximum level of exposure supposed to kill foragers.

Semi-field and field studies are designed in order to assess the effects at the colony-level, including all bee categories (cast levels), under conditions representative of the proposed use. The assessment of effect consider mortality, foraging behavior, and other effects on the colonies. If pollen or nectar containing residues are brought back to the hive, colonies should be monitored during a sufficient time period to also check long lasting or delayed effects on parameters such as brood development or queen health.

For both semi-field and field trials, it should be demonstrated that the test bees were exposed under the environmental conditions (especially weather conditions in the case of field trials) of potential exposure. Parameters such as pollen collection, residue analysis, as well as flight intensity, and observation of the activity on flowers of the treated crop are useful information.

In cases where observations from semi-field or field studies indicate effects on the colony as a result of an exposure to treated fields, further investigation may be envisaged with the aim to further characterize the conditions of occurrence of effects. As an aid to risk management, additional testing conditions may be incorporated into cage or field trials, in order to examine whether adverse effects can be reduced by changing the conditions of use (e.g. lower application rates or changing the timing of application in relation to flowering).

Overview and Status of Ecological Risk Assessment for Pesticides. Canadian Perspective
Bob Sebastien, Pest Management Regulatory Agency (PMRA), Health Canada

PMRA relies on a risk assessment process similar to that of the U.S. EPA. It is a tiered process that consists of evaluating risk through an assessment of both exposure and hazard.

The exposure assessment identifies the chemical/physical properties and environmental fate properties (persistence, transformation, and mobility), and the use of a compound. The hazard assessment identifies the acute and chronic toxicity of the pesticide (parent compound) and its degradates of concern. Studies are also required to assess whether a compound has potential to bioaccumulate. The fate and effects of the compound are then integrated in the risk characterization to identify environmental concerns, and risk management explores potential means to mitigate risks.

PMRA relies on acute contact and/or acute oral toxicity tests with honey bees; these studies are based on EPA and Office of Economic Cooperation and Development (OECD) protocols[5]. Depending on the outcome of the acute oral[6] and contact[7] toxicity tests, compounds are grouped into one of the three following categories.

[5] OECD 2010. Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems. http://lysander.sourceoecd.org/vl=3912267/cl=16/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n2/contp1-1.htm
[6] OECD Guidelines for the Testing of Chemicals. Test No. 213: Honeybees, Acute Oral Toxicity Test http://lysander.sourceoecd.org/vl=3912267/cl=16/nw=1/rpsv/ij/oecdjournals/1607310x/v1n2/s14/p1

Group 1 - Highly Toxic**:** LD_{50} 0.001-1.99 µg active ingredient (a.i.)/bee. Severe losses may be expected if the compound is used when bees are present at treatment time or within a few days thereafter.
Group 2 - Moderately Toxic**:** LD_{50} 2.00-10.99 µg a.i./bee. These can be used around bees depending on dosage, timing, and method of application, but should not be applied directly on bees in the field or at the colonies.
Group 3 - Relatively Nontoxic**:** LD_{50} >10.99 µg a.i./bee. Pesticides in this category can be used around bees with a minimum of injury.

To assess hazard, PMRA requires hive/brood studies for insect growth regulators to determine if adult or brood exposure to residues in pollen/nectar leads to adverse effects. These studies include semi-field or field studies. Measurement endpoints include the numbers of foraging bees; mortality of foragers; pollen collection; number of bees in hive; brood status in frames; and residues in pollen, bees, wax and honey. PMRA employs the various protocols that are available (e.g., EPPO Guideline on test methods for evaluating the side-effects of plant protection products on honeybees, EPPO Bulletin 22, 203-215 [1992]).

For contact exposure assessments, the cumulative seasonal application rate (maximum rate x number of applications) is used, based on the measured foliar dissipation rate or a 35-day default value. For systemic pesticides, exposure is calculated by considering residues in pollen and nectar along with consumption rates for these items.

For risk assessments, the median lethal dose (LD_{50}) in micrograms of active ingredient per bee (µg a.i./bee), from the contact toxicity test, is converted to the equivalent number of kilograms a.i. per hectare resulting from an aerial application by multiplying by 1.12 (Atkins *et al*. 1981). For example, an acute LD_{50} of 100 ug a.i./bee would equate to an application rate of 112 kg a.i./ha that would be expected to kill 50% of the bees foraging in the treated field at the time of application or shortly afterwards. If the proposed label rate is 0.1 kg a.i./ha, then a risk quotient of 0.001 can be calculated (0.1/112 = 0.001). Pollinators would therefore be at negligible risk following an application of this pesticide.

For systemic insecticides where residues are expected in nectar and pollen, empirical data on the concentration of residues in pollen and nectar can be factored into literature-reported values for consumption of pollen and nectar by foraging bees to calculate a daily dose. The daily dose consumed is then divided by the acute oral LD_{50} to obtain a risk quotient for oral exposure. Similar to the U.S. EPA assessment scheme, the focus of this assessment scheme is the adult female forage bee, and does not consider other adults (male drones, young nurse bees, queen) or developing (brood, larvae) bees. Depending on the outcome of the acute oral/contact toxicity test, bee warning statements may be required on the pesticide product label.

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[7] OECD Guidelines for the Testing of Chemicals. Test No. 214: Honeybees, Acute Contact Toxcity Test. http://lysander.sourceoecd.org/vl=3912267/cl=16/nw=1/rpsv/ij/oecdjournals/1607310x/v1n2/s15/p1

Data and Information Needs:

Exposure from Systemic Compounds: What is the exposure to pollinators from persistent, systemic pesticides where residues can be translocated from soil or treated seed into plant nectar and pollen?

Sublethal Endpoints: What sublethal endpoints are appropriate for risk assessment for compounds that effect the central nervous system?

Exposure Duration for Testing: What is an appropriate exposure period required to characterize the risk of these types of compounds?

Uncertainty around Mitigation for Systemics: Exposure to systemics is uncertain and developing mitigation (if necessary) may be difficult for systemic compounds.

Lab-to-field Data: The relationship between laboratory studies and field effects needs to be better understood.

Overwintering Testing: Current test procedures do not consistently assess the overwintering period of colonies in hazard studies.

Overview and Status of Ecological Risk Assessment for Pesticides—a State's Perspective

Rich Bireley, California Department of Pesticide Regulation (DPR)

California's Department of Pesticide Regulation (DPR) has limited resources and does not conduct formal ecological risk assessments, as does the U.S. EPA. However, California regulations require DPR to investigate reports of possible adverse effects to people or the environment resulting from the use of pesticides. If a significant adverse impact occurred or is likely to occur, the regulations require DPR to reevaluate the registration of the pesticide. The process is formal and may be initiated based on review of data submitted by a registrant (e.g., chemical company), other government agency, or the public. The common triggers include public or worker health hazard, environmental contamination, or adverse effects to nontarget organisms. Once initiated, DPR may require registrants to provide data. Failure to provide the required data can result in cancellation of pesticide products. The reevaluation data requirements are specific to the observed adverse effect. Possible outcomes of a reevaluation include the following: 1) no label change or mitigation required; 2) mitigation measures are needed, such as new regulations, permit conditions, or label mitigation; or 3) the adverse effect cannot be mitigated and the pesticide or specific uses must be canceled in California.

With regard to honey bees, DPR relies on acute oral and contact toxicity studies as well as any other (semi-field/tunnel; field) that may be available. The DPR evaluation process is intended to identify chemicals of potential concern because of likely exposure and effects. DPR requires companies to submit any data that indicates a potential adverse effect, and reviews studies required by EPA as a condition of registration for use in the State of California. Based on the analysis of available data, DPR may suggest label mitigation or decide not to register the pesticide in the State of California.

With respect to the neonicotinoid insecticide imidacloprid, DPR has received letters expressing concern for honey bees over the use of pesticides containing these products, which are registered for numerous uses, including commercial crops, landscape maintenance, and home use. Many products are applied directly to the soil, and DPR notes that claims regarding the duration of efficacy (*i.e*., duration of time the chemical remains effective against target pests) have been increasing. Additional data on imidacloprid recently submitted by the manufacturer of the technical grade product have demonstrated the persistence in plant tissues and at levels sufficient to result in adverse effects to beneficial insects such as bees over prolonged periods of time. Based upon information received, estimated dietary LC_{50} values (for imidacloprid) in honey bees are exceeded by residues found in plants. Therefore, DPR has placed imidacloprid and three other neonicotinoid pesticides into reevaluation as described above and is requiring additional data from the registrants.

For each individual neonicotinoid as identified in the reevaluation (imidacloprid, dinotefuran, clothianidin, and thiamethoxam), DPR proposed to require an acute larval toxicity study as well as an acute oral LD_{50}. DPR will allow the registrants to conduct the required studies or use a similar neonicotinoid--which DPR has designated as imidacloprid. Acceptable study protocols must be submitted to and approved by DPR before the studies are initiated. Depending on the results of these studies, DPR may require additional testing, including a chronic bee study, residue analysis study of honey, and greenhouse or field toxicity studies.

Data Requirements for the DPR Neonicotinoid Reevaluation include
Field monitoring residue studies of nectar and pollen collected from crops where the insecticide had been applied for two consecutive seasons.

Proposed acute toxicity studies included acute dietary toxicity studies to provide an estimate lethal concentration (LC_{50}) to 50% of the larval honey bees tested

California has a particular interest in preserving honey bees, since almond production in the State produces approximately 80 percent of the world's supply. Currently, almond pollination requires 1.5 of the 2.5 million commercial honey bee colonies in the U.S.

Summary of Current Toxicity Testing
Mike Beevers, California Agricultural Research (CAR)

CAR conducts studies to measure the effects of potential exposure levels, as well as to examine frequency and intensity/duration of exposure. Although guideline studies are relied upon for risk assessment, non-guideline studies are sometimes required and conducted on a case-by-case basis.

Dose determination questions include:
- Are the test animals actually eating the test substance?
- Are the nurse bees removing the test substance?
- Which positive controls (arsenic or low rates of dimethoate) should be used?
- To what extent does the environment influence test outcome?

The physical characteristics (solubility, palatability, stability, etc,) of the test substance can affect the study. In some studies, it is difficult to quantify how much test substance is consumed per bee. The CAR lab is currently evaluating an *in vitro* larval test designed by Dr. Zachary Huang to improve larval/pupal survival of bees through emergence as young adult bees.

One disadvantage of the current tiered process of testing is that it does not necessarily reflect potential colony level risks, since it doesn't reflect the in-hive processing that occurs with pollen/nectar. Another consideration is the possibility of sublethal effects and their effects on the next generation of bees.

Part 2: Panel Discussion

The Panel considered how sublethal effects should be measured and incorporated into an ecological risk assessment. Tier 1 studies focus on acute toxicity endpoints (e.g., survival lethality), whereas higher tier studies consider chronic endpoints such as growth, survival and reproduction. Given the focus of Tier 1 and higher-tiered studies, an assessment of sublethal effects should be regression-based for acute toxicity tests and hypothesis-based for chronic tests. This is to say that acute effects should be expressed as a regression-based EC_x (the concentration resulting in an adverse effect to x% of the organism tested) while chronic study endpoints are expressed as a hypothesis-based no observed effect concentration (NOAEC) and the lowest adverse effects concentration (LOAEC) In considering sublethal (and acute) effects, regulatory agencies should evaluate the potential ecological relevance (*i.e*., ecosystem-level impacts on growth, reproduction, and survival). Sublethal effects are more likely to be seen from low-level exposure studies than acute (high-dose) exposure studies. Therefore, any biological expression of sublethal effects are addressed in higher tier studies; however, sublethal effects may not be clearly dose dependent, but may be sporadic, further confounding efforts to link sublethal measurement endpoints with regulatory agency assessment endpoints for risk assessment.

Considering the relationship between the acute laboratory studies and the field studies, panelists noted that the individual (single forager) bees behave differently from the colony; although dose dependent studies of individual [caged] bees can provide reproducible results on individual bees, it is questionable if these results relate to colony-level effects. This phenomenon further complicates design and interpretation of data. For example, proboscis extension reflex (PER) is only conducted/tracked in laboratory studies since this measurement endpoint cannot be tracked in the field. As such, measurement endpoints such as these may have limited utility. The focus therefore is typically on impaired individual honey bee and colony survival, growth, and/or reproduction.

Because of the variables that occur with toxicity tests, the panel members identified a number of additional remaining questions, including but not limited to:

- What constitutes an appropriate dosage rate for the individual bee and/or the colony?
- Are honey bees the best surrogate for pollinator testing or should native bees and other pollinators also be tested?
- Should laboratory acute tests be extended beyond the normal 48-96 hour time frame, *e.g.* 20 days or longer, to fully evaluate reduction in adult fitness (longevity)?
- Can individual effects be related to hive/colony health?
- Should behavioral effects be assessed when evaluating acute study results (on hive health)?
- Neurotoxic effects on individuals are easy to measure, but how does it impact the colony?
- How should sublethal effects at individual and colony level be defined and distinguished?
- How much larger should sample sizes be to improve test power?

- How are the effects of volatiles on larvae evaluated?
- Accumulation of pesticides in comb wax could be a problem. Should this be evaluated in-field?

LARVAL ACUTE TOXICITY TESTS: LABORATORY

Part 1: Presentation Summaries

Laboratory Larval Acute Toxicity Testing Methods
Zachary Huang, Michigan State University

Alternatives to in-hive testing include *in vitro* techniques. *In vitro* testing techniques are, in part, designed to reduce environmental factors. Dr. Huang noted that larval tests are conducted at 35^{o}C and 95% relative humidity to mimic conditions in the hive, and that it is important to recognize when brood defecate, otherwise the larvae will consume their excrement. This is why larvae must be transferred into a new Petri dish to pupate. In lab testing of various diets to improve survival, beeswax-coated cells decreased survival.

The protocol used by the presenter allowed for 75 - 85% survival from first instar larvae to adult emergence and is currently undergoing ring testing in Europe. In tests conducted by Dr. Huang, no significant effects on honey bee survival were noted by E64, an irreversible inhibitor of cysteine proteinases, however, there were significant differences in adult emergence weight and developmental time. Unexpected effects noted by Dr. Huang suggest the need to evaluate these endpoints, in addition to mortality rate differences.

Larval bee studies present challenges in providing the appropriate environmental conditions to insure reasonable survival, growth and development of the test animals. *In silico* methods used at Pennsylvania State University (PSU) have demonstrated a 10% loss at larval stage and 10% loss in pupae stage. Once the developing bee has reached the white-eyed pupal stage, the likelihood of mortality substantially drops. Additionally, a clear definition of what constitutes the experimental unit, *i.e*, whether it is the entire multi-well plate or the individual cells within the plate, is needed in these studies. Typically, weight measurements are based on individual bees but survival is based on the plate which confounds efforts to define the experimental unit.

Panel members expressed concern that considering the individual bee per well as the replicate would constitute pseudo-replication. Concern was also expressed about pathogen growth on the developing larvae/pupae. However, the protocol relies on the use of media that minimizes pathogen growth. Therefore, it is important to better define the environmental conditions under which the studies should be conducted to generate data and the statistical methods that should be employed to analyze the data.

Summary of Current Larval Testing
Mike Beevers (CAR)

CAR conducts a test on larval development that is sometimes referred to as an "in hive" test. That is because frames are removed from the hive, and individual 2nd in-star larvae are dosed in their cells. These larvae, in cohorts of 20 per treatment, are then allowed to feed for a half hour and the frame is then returned to the hive and development monitored through adult emergence. Test frames are selected based on brood presence and hive health. After exposing larvae to treatments, frames are returned to the hive for capping by nurse bees. Frames are removed from hives again to evaluate capping and then returned to hives until adult emergence. Once all cells are capped and adult emergence is near, frames are placed in a growth chamber and cages are placed onto comb to capture adults as they emerge. Daily observations of adult emergence are recorded.

Considerations for discussion and future testing include: dose determination; positive controls; and, appropriate exposure scenarios to ensure larvae are feeding on the test substance. Physical characteristics (*e.g*. odor) and palatability may affect uptake of the test substance. Sublethal effects on adults should also be considered.

Part 2: Panel Discussion

In a tiered testing approach, such as that employed by EPA for chemical pesticides, Tier 1 acute toxicity studies focus on contact toxicity to adult honey bees. However, an issue related to this type of testing is that adult bees move around the hive, which increases the chance of cross contamination. Therefore, panelists suggested that larval testing be considered as part of Tier 1 or Tier 2 testing, since the test organism remains in the cell and can be easily studied in their natural environment. Panelists also noted that when deciding whether a larval or adult acute toxicity study is appropriate, the life stage (*e.g*., larvae, nurse bee, forager) that has the greatest potential exposure to the pesticide product and susceptibility should be the focus, and one panel member suggested that this most highly exposed member may be the nurse, since it is these bees that expose larvae through feeding. Other members ,however, noted that adult (foraging) bees may be more highly exposed than larval (or the nurse) bees.

Larval testing is appropriate in many cases since:

- contamination is less of an issue;
- the larvae are more sensitive to exposures than adults, and
- their stationary nature obviates many behavioral challenges.

However, oral tests with larvae are more appropriate than dermal exposure studies. Panelists also noted that larval testing may be appropriate when evaluating systemic pesticides but that it is not the best assay for a contact pesticide.

There was some discussion among the panel of whether the (larval) tests reflect "real world" contamination levels to which bees are exposed. Participants discussed the purpose of "standard protocols" and whether contamination of test material was appropriate in standard test practices. Although testing clean hives is not representative of what will occur under normal field conditions, developing protocols which standardize test materials and conditions will eliminate variability and "background noise" that otherwise make results difficult to interpret. Further, if hives with other contaminants (*e.g.*, the miticides coumaphos and fluvalinate) are used in a test, the control should indicate if any observed effects are treatment related or not. Ideally, a study would be conducted according to good laboratory practices (GLP) and would utilize the cleanest possible environment (*e.g.*, new frames) and analyze wax and royal jelly to ensure other chemicals are not present. Also, a screening-level assay could be used to invalidate a test if background levels of pesticides or contaminants are detected. In addition, standardizing bees used in research should be considered to account for genetic diversity. Other indicator organisms should also be considered since there is wide genetic variability in the European honey bee *Apis mellifera*.

One panel member noted that a tiered testing approach may not work since the acute (larval) test does not reflect the complexity of the colony or of the field; a matrix of lab and field studies may be more appropriate. A full field test is not always feasible or cost effective. Therefore, a testing step between acute toxicity and field testing (*e.g.*, semi-field) needs to be defined and routinely used.

It was also suggested that, rather than only testing individual chemicals, formulated products that contain multiple active ingredients and inert ingredients should be tested at semi-field level in order to reflect real interactions and account for potential synergistic or additive effects from exposure from multiple stressors.

ADULT ACUTE TOXICITY TESTS: LABORATORY

Part 1: Presentation Summary

Adult Honey Bee Study Design
Jeff Pettis, USDA, Agricultural Research Service (ARS), Beltsville Bee Research Laboratory

The use of laboratory adult acute toxicity tests is common, in part because the test is highly controlled. Field studies, unlike contact laboratory studies, have more drawbacks due to the use of multiple colonies that add variability to field studies, but have the potential to be more informative. Currently, however, acute oral studies are not required on a routine basis by EPA.

EPA does require acute contact testing, and the parameters that should be considered for a valid acute contact toxicity test include the following.

- The solvent alone should always be used as a control; when testing a dust formulation the carrier should be used as a control.
- The time intervals for mortality assessments should include measurements at 4, 24, and 48 hours post-treatment.
- The genetics of the bees in the tests should be from a minimum of six genetically diverse colonies.
- Twenty five bees per dose should be tested at five or more dosage levels. This range in dosages allows for probit analysis to be conducted and a valid LD_{50} value calculated.

- A test would be considered valid if less than 20% mortality occurred in the control bees. Control mortality above 20% indicates problems in bee handling or experimental setup that call into question the validity of the test.
- Residue on foliage studies typically involves a cage study utilizing 25 bees/cage with 6 cages/treatment. Typically, plant foliage (preferably alfalfa) is left in a cage where exposure (dermal and presumably some oral) occurs. In future studies, it may make sense to have a whole plant in the cage, *i.e.*, roots as well as foliage, which may allow for other routes of exposure to be assessed. Dietary exposure is presumed to occur when the bee cleans itself.

Pettis noted that EPA historically relies on acute contact toxicity tests with young adult forage bees and may require acute oral toxicity tests on a case-by-case basis. In briefly discussing field tests for pollinators, Pettis noted that the frequency at which EPA requires field testing is dictated by results from lower tiered studies, and whether additional lines of evidence (e.g. beekill incident reports or open literature showing effects on honey bee colonies) compel EPA to require higher tiered testing.

Generally, acute contact toxicity studies are not adequate to assess the potential toxic effects of systemic compounds that may be present in pollen and nectar. Therefore, acute oral toxicity studies should be run in parallel to any acute contact studies in these scenarios. The results of the two studies (*i.e.*, acute contact and acute oral) can then be compared relative to the mode of action of the test chemical.

Pettis noted that for an acute oral toxicity test, some consideration should be given to extending the current test duration of 4 hours to 10 days (that typically used by European regulatory authorities) to 20 days. It is reasonable that measures of mortality and sublethal effects could be recorded at various time points (*e.g.*, 48- and 96-hr) throughout the duration of the study. He also noted that consideration should also be given to using spiked sucrose solution or spiked bee bread (pollen substitute) as an exposure media provided that residue levels in these exposure media are accurately quantified. An adult oral toxicity laboratory test should be conducted on a case-by-case basis, when conditions mimic actual use pattern, and the pesticide is applied at the maximum label rate and frequency (minimum reapplication interval).

Additional considerations include:

- Cage foliage studies may not be realistic and representative of potential risks.
- The panel noted that replication in chronic tests is important, but costly.
- Use of LC_{50} may be more appropriate than LD_{50} for many chemicals. Would different results be expected using the different determinants?
- Using time to lethality at high dosages is a rapid and cheap way of screening.

Part 2: Panel Discussion

Tier 1 adult acute contact toxicity studies are routinely conducted for synthetic pesticide compounds that are intended for outdoor use. However, the panel agreed that the more realistic acute oral toxicity assays should also be required for risk assessments on a case-by-case basis, particularly for systemic pesticides. Possible criteria for requiring oral toxicity tests include:

plant uptake, formulation type, method of application, and mode of action (however, often times information on the mode of action is not available and is not part of the required data).

When designing an oral toxicity assay, the dosage should be based on potential exposure to the pesticide from feeding on pollen and/or nectar. This can be done by determining the concentration in the whole plant (foliage) and extrapolating to pollen or nectar as is done in Europe.

As for test length, the panel discussed that it may be beneficial to conduct the acute test for the entire life span of the bee, beyond the typical 96-hour study, to evaluate sublethal effects. These longer tests may be particularly useful for lower doses of pesticides.

Panelists also noted that conducting tests with summer bees may be preferred. While larvae may be the most appropriate stage to test for certain compounds (*e.g.*, insect growth regulators [IGRs]), young adult (female) bees, *i.e*, nurse bees, are the most likely exposed to other types (systemic) of compounds or residues on pollen and nectar.

Test size was also an important item of discussion. Current test designs specify a sample size of 25 bees; however, it is uncertain if this is a large enough sample size for statistical validation. As a test with adequate statistical power cannot be designed without an understanding of what magnitude of an effect (for a given endpoint) would be considered adverse, regression-based testing was suggested as a way to get confidence of a dose response curve with few repetitions. A higher number of replicates would be needed for a chronic toxicity test in an attempt to mimic field conditions and detect treatment-related effects with a specified level of certainty. It was noted that it is not practical to include sufficient replication in a field test to assure enough statistical power for effects to be determined at treatment levels.

Dose response tests may also be required, which can be determined from a limit dose test. For a limit dose test, a bee would be exposed to an expected maximum [estimated] exposure value for the test substance. No additional testing would be needed if no mortality is recorded at the maximum estimated exposure level. However, if greater than 50% mortality was observed in the limit test, a dose response test would be required..

For an oral toxicity assay, exposing the bees to the pesticide through sucrose solution is preferred to bee bread because the bees consume all of the test substance, and it is easier to standardize a test [quantify exposure] using sucrose solution. A protein supplement is recommended when bees are dosed via a sucrose solution. A "spiked" pollen substitute may be fed to the bees that will be consumed within approximately three days; however, it is difficult to determine if all bees are exposed to the same amount of test substance. Hypopharyngeal gland development can be used as a measurement of a potential effect when the bees are fed the pesticide with pollen.

Panelists also discussed appropriate endpoints of an acute toxicity test. Though these are typically reported as a $LD_{50,}$the panel believes this is only appropriate for a contact toxicity test. To report oral toxicity as an LD_{50}, the dosage, *i.e*., the quantity of material consumed by each organism, would need to be quantified. Since it is difficult to quantify the amount of the toxin that is ingested by a test organism, it is more appropriate to report results as an LC_{50} for oral toxicity tests.

It is unclear if a Tier 1 contact or oral toxicity test using adult honey bees is needed for a risk assessment. The Panel recommended conducting a meta-analysis of existing data based on mode of action, which may help determine which type of test is needed. The possibility of using genetic markers was also considered, but biological markers more clearly linked to apical endpoints of impaired growth, survival and/or reproduction were considered better determinants.

The Panel identified the following as areas needing further investigation:

- Is a Tier 1 adult contact toxicity test appropriate for systemic pesticide risk assessments? If so, should assays be conducted in small hives?
- How representative are the laboratory tests [on individual bees] of colony effects and do these effects extend to field conditions as well?
- An artificial diet needs to be developed for larval assays because royal jelly may have other contaminants.
- Variation within *Apis mellifera* needs to be characterized to determine if race or breeder strains matter and if a test is repeatable with variable bees. Characterize genetic variation of test bees and relate to susceptibility (need to understand how *A. mellifera* represents other species of bees).
- How should disease pressure be assessed after pesticide exposure?
- What are the sublethal impacts of pesticides (*e.g.*, AChE inhibitor)?
- Indicator species – can the honey bee be a surrogate for insect pollinators or as a surrogate for terrestrial invertebrate, and are these appropriate benchmark organisms?
- Enhanced protocols must be developed to assess how pesticides impact colony dynamics
- Artificial diets.
- Mode of action – sublethal effects should be assessed (target site versus sensitivity of system as a whole; how many neurohormones are affected?).
- Formulation tests need to be developed
- Researchers need to look at synergistic effects to determine “real work” exposure scenarios at the field scale
- Individual effects on hive/colony should be assessed.
- Standardized experimental protocols such as pollen substitute, syrup, and cage need to be developed.
- Studies are needed on "flow"/transfer of pesticides through colonies.
- What is the source of bioaccumulation/transfer of pesticides (how does pesticide move through colony, *e.g.,* pollen versus nectar, and which caste/stage of bees gets the greatest exposure)?
- Gene expression studies are necessary (use of “omics” data has to be related to apical endpoints of survival, growth and reproduction).
- Studies are needed to determine the level of residues in pollen and nectar to determine exposure.
- Research is needed to evaluate metabolites/degradates/transformation products and how they translocate in the plant- particularly for systemic pesticides.

One panel member noted that much data currently exists, and in particular, data from the acute toxicity test for positive controls (*e.g.* dimethoate) may provide insight on the impact of genetic variability. This larger data set could also provide insights on how sublethal effects in acute studies relate to what was seen in chronic studies.

CHRONIC TOXICITY TESTING: LABORATORY

Part 1: Presentation Summary

Summary of Chronic Test Methods
Mike Beevers, California Agricultural Research (CAR)

As a contract research lab providing registrants with honey bee regulatory tests for EPA submission, CAR conducts chronic toxicity tests in the laboratory. Hive history is recorded and studies are conducted according to Good Laboratory Practice (GLP) under controlled temperature and humidity. The study uses four replications of 50 newly emerged bees per cage, which are fed a sucrose solution with no pollen. A standard positive control such as arsenic or low dose of Dimethoate is used to validate exposure and the ability of the test to detect treatment-related effects. Each cage of bees is provided with 10mL of test solution every other day.

This type of study is consistently repeatable and has high untreated survival. In conducting this test, however, problems sometimes arise in determining the dose required to accurately reflect environmental exposure and test substance solubility and stability in a sucrose solution food source. In addition, it is difficult to meet current guidelines recommending that the study be conducted for 30 days, as it is difficult to consistently rear adult bees for that long.

Part 2: Panel Discussion

Laboratory studies that evaluate potential toxicity to pollinators from chronic exposure to pesticides are not routinely conducted in the U.S., but are conducted in the European Union (EU). A typical study in the EU includes a 10-day sucrose exposure test conducted with caged bees using mortality as the measurement endpoint.

The Panel discussed criteria to consider in developing a standardized laboratory chronic toxicity test protocol with bees, such as exposure scenario, duration, and endpoints. Criteris for exposing bees to a pesticide in the laboratory should be through a mechanism relevant to real world field exposure. One such mechanism is through a spiked sucrose solution, which is preferred to honey because it results in fewer variables that may confound the study (such as existing pesticide residues). When exposing bees through a spiked sucrose solution, cane is preferred to beet sugar, since beet sugar has been found to cause diarrhea in bees.

Bees may also be exposed to a pesticide through feeding on spiked protein especially when exposure is likely through pollen feeding. Protein supplements are preferred to pollen because pollen brings other contaminants (*e.g.*, certain plants incorporate flavonoids into pollen which may be toxic to bees), and protein content varies between pollen-types; pollen may be used when considering a specific crop. Panelists noted that it is difficult to control the dose with spiked protein because continuous exposure to spiked sucrose may lead to foragers storing material in the hive. In addition, protein is needed in the diet in order to monitor hypopharyngeal gland development. The feeding of spiked protein and or syrup both have limitations and the choice of one over the other may depend on the environment in which the study is conducted and the availability of competing nectar and pollen.

Bees are not just exposed by consuming protein; they can walk through the protein mix and have contact exposure and possible inhalation exposure as well. If feeding protein, then bees will have to defecate, which is not the case when using sucrose; and, while bees will not typically defecate in the hive; confining bees to the hive may result in such behavior. As such, contact with the contaminated waste would represent an additional route of exposure that may be difficult to quantify.

An alternative approach to obtaining chronic toxicity information is to adapt an acute contact (topical) exposure study, which is typically conducted for 48 hours, and prolong the observation period to follow survival for 21 days. Possible endpoints of a chronic study include, but are not limited to, adult longevity, glandular development, immune response, pathogen challenge (*e.g.*, challenge with Nosema and count spores in gut), or emerged bee weight. Sources of variation need to be identified in order to minimize their effects. Studies can be done to quantify variation and data may be used for future modeling.

Other considerations include anesthetics, temperature, and materials used in the study. Panelists agreed that further consideration should be given to the use of CO_2 to anesthetize bees, although the use of day-old bees unable to sting is preferred,, because CO_2 will decrease the life-span of bees and causes changes in juvenile hormones. Temperature is also variable among studies. Current studies vary in temperature from 25°C to 34°C, and studies are most commonly conducted from 30°C to 34°C; other temperatures seem too low since wing development is affected and bees don't fly at 23°C. When testing larvae, a slightly higher temperature (*e.g.*, 35°C) may be needed. Cage size and material (preferably new material) as well as bee density also need further consideration.

CHRONIC TOXICITY TESTING: SEMI-FIELD STUDIES

Part 1: Presentation Summary

Semi-field Study Design
Mike Beevers, CAR

Semi-field studies involve the use of outdoor mesh tunnels covering a small individual hive and a blooming foraging source. This has the advantage of isolating the food source for the hive. By treating the food source, the effects of treatment can be assessed. There is a limited amount of time that a hive of bees can survive in the tent (previous studies have shown that it is difficult to keep hives in a tent for 7 or more days). The correct stage of crop bloom is critical to the study.

Treatments should be applied before bees start foraging. Nuc hives with adequate eggs and larvae should be used. Hives should be acclimatized prior to test initiation. Hives are under stress, particularly from heat. It is wise to limit the number of times a hive is opened. Water must also be provided.

Various endpoints can be measured from semi-field studies. Percentage of the comb with brood, pollen, and honey as well as larval development may be used as indicators of colony health. Foraging is assessed by counting foraging bees per square meter for 30 second intervals. Numbers of dead bees and entrance activity can also be measured.

Part 2: Panel Discussion

In Europe, semi-field studies are conducted as compliment to other tests. Under the EPA's Office of Pesticide Program testing paradigm for terrestrial invertebrates, higher tier tests such as a semi-field or field study are triggered on a case-by-case basis if prolonged foliar residue is expected, or if open literature indicates effects to pollinators and if lower tier testing indicate acute contact toxicity of <11 ug/bee. Field studies may also be needed for a new chemistry with little to no data or an old chemistry where no data exists. Additional situations that may trigger the need for a field study include a pesticide with potentially high use on many flowering crops, incidents reported from a pesticide in use, or degradates that are potentially toxic.

Semi-field tests often report good survivor rates and measurable exposure, and endpoints (such as foraging rate) are often easy to measure. However, conducting studies in tents or tunnels may lead to high stress in colonies due to limited food and the need for small size colonies. *Osmia* bees and bumble bees may be more workable than honey bees in tent studies since these pollinators do not forage far.

Although the primary endpoint measured is mortality, cohorts of eggs, larvae, and capped brood may also be appropriate endpoints to consider. This may be accomplished by marking 100 open cells to be checked 3 to 5 times to assess 2-day old eggs and 4-day old larvae.

CHRONIC TOXICITY TESTING: FIELD STUDIES

Part 1: Presentation Summaries

Field Study Design Elements
Galen Dively, University of Maryland

A "field study" is generally defined as an open field experiment that involves functional colonies exposed to a pesticide under actual field conditions. The purpose of a field study is to assess lethal and sublethal exposure effects on the pesticide and bee colony health, performance, and foraging behavior under typical use conditions. The focus of a field study is on the health and strength of the colony, rather than the individual bee.

Two possible exposure study scenarios were presented by Dr. Dively. The first involves colonies placed in or on the edge of large treated fields of flowering crops. Replicated treatment and control fields should be separated and isolated as much as possible from other sources of pollen and nectar. Ideally, treatments are applied at full bloom when bees are active. Pollen is collected using pollen traps at the hive entrance to analyze for residue content and whether the pollen was collected from test fields. Before and after treatment, researchers measure endpoints to evaluate potential effects.

Another scenario involves exposing colonies to a known concentration of the pesticide via in-hive feeding with treated pollen diet or sucrose water. Replicated treatment and control colonies are placed together in an apiary and isolated as much as possible from flowering crops treated with the same pesticide. Colonies are exposed to single or continuous exposure regimes of different concentrations of a treatment, plus negative and positive controls. Various endpoints of effect are measured before and after treatment.

Generally, field tests involve nucleus (nuc) hives with five foundation frames with all new equipment. For one month, package bees with sister queens are fed sucrose water to build up colony strength. Colonies should be equalized prior to exposure and placed in at least five bee yards in different locations. Colony performance can be measured from several measurement points:

- percent of comb area with bees; capped brood; late larvae; bee bread, honey; presence of eggs;an active queen; number of foragers with and without pollen pellets; weights of foraging bees and pollen loads
- pollen diet consumption
- weight of pollen collected.

Other endpoints that should be considered for evaluation include overwinter survival, size of surviving cluster, rate of colony buildup the following spring, and colony performance effects from repeated exposure treatments the second year.

Field Study Design Elements
Richard Rogers, Wildwood Laboratory, Nova Scotia, Canada

Richard Rogers noted, as did previous speakers, that a field study may be required for honey bee risk assessment for various reasons. When designing such a study, many factors affecting bee health (*e.g.*, environment, shelter, and safety) need to be considered. Therefore, in designing the size of a study, the benefits of increased size (to reduce variability and increase statistical power) must be weighed against the advantages of decreased size (to manage and track).

Ultimately, test design will vary depending on the endpoint being measured. Endpoints that can be measured from field studies include percent of comb area with bees, capped brood, late instar larvae, bee bread, and honey; presence of eggs and active queen; number of foragers with and without pollen pellets; weight of foraging bees and pollen loads; pollen diet consumed; weight of pollen collected; overwinter survival; size of surviving cluster; rate of buildup in spring; colony performance following repeated exposure in second year; brood cohort success; colony strength and success; pupae and emerged bee weight; nectar and pollen residues; hive product (wax, honey) residues; bees on crops; incoming workers; colony strength/health; and foraging behavior.

Part 2: Panel Discussion

In discussing the benefits and disadvantages of field studies, panelists indicated that they more closely represent "real world scenarios" than laboratory studies, but are more difficult to control. Field studies typically rely on functional hives and exposure scenarios representative of actual field conditions. Field studies are intended to measure lethal and sublethal effects and assess endpoints such as hive health and foraging behavior, unlike laboratory studies, which assess effects on individual bees. Presenters also mentioned that bees used in field studies can be used for other related studies afterward, unlike for laboratory studies.

The panel discussed two exposure scenarios for field studies. First, colonies are placed on the edge of a large field where the field is then treated at full bloom when the bees are active (exposure is verified by examining collected pollen). The difficulty is limiting the extent to which bees may forage off the treated field; therefore, treatment and control fields need to be adequately separated. Alternatively, colonies can be exposed via in-hive feeding using spiked sucrose or pollen. In the latter case, treated and control hives can be kept together.

Ideally, full hive studies should be kept simple with a single stressor exposed to nucleas (nuc) hives (5 foundation frames), package bees, and sister queens. A drawback is that if the study is prolonged, bees may become crowded leading to potential swarming. Prior to initiating a field study, hives should be built up for one month by feeding them on sucrose water, and equalized prior to testing and new equipment is preferred. Ideally, hives would be established from packaged bees so their expansion and hive invasion can be observed. However, most studies use existing bees because packages need to be ordered months in advance.

Large plots or semi-field studies should be used for chronic toxicity testing in the field. When selecting plots to conduct field studies, the crop being investigated needs to be considered as well as knowledge of bee's foraging radius. Understanding competing vegetation in the area can help limit off-field foraging. Trapped pollen can be analyzed to determine what crops the bees use as forage. Cage or tunnel studies may be preferred over field studies due to limitations such as low numbers of colonies per plot, small percentage of foraging radius, and the fact that monitoring takes place only during bloom.

As an example of a field pollinator study, panelists discussed a project on watermelons conducted in conjunction with USDA, University of Maryland and U.S. EPA, included free foraging bees exposed to imidacloprid at 0, 5, 20 ppb in Megabee® protein diet supplement for 9 weeks (approximately 3 brood cycles); colonies were fitted with pollen traps to better ensure that study bees would consume the spiked pollen inside each of the colonies. Pollen combined with honey incorporated into "bee bread" was used to further ensure that bees ingested the spiked pollen. A 100 ppb treatment was added to serve as a positive control. As colonies expanded during the study, frames were added to the hive. Dively indicated that hives should not be manipulated too often since it will stress the bees and could increase drift.

Exposure to a pesticide can be evaluated from pollen, nectar, and wax measurements before and after exposure. Field studies that include dead bee traps can be used to measure potential mortality from exposure to a pesticide in a natural environment. Other endpoints that should be routinely analyzed by field studies include queen status and returning pollen foragers. Additional parameters that can be measured include pest and disease levels and the number of drone cells; colonies under stress do not produce drones. However, the number of parameters sampled is less important than the number of colonies; more colonies are preferred when possible.

Future Research

Additional research is needed to determine how to design an optimal field study. In the future, researchers should consider the following:

- Determine how studies expose bees and at what residue levels
- Develop an in-vitro assay for immunological stress and detoxification activation
- Devise parameters that can be used in a model
- Develop sampling techniques for other pollinators and pollinator diversity
- Develop genetic markers for bee fitness
- Determine which parameters highlight risk
- Determine when results from a field test prompt action
- Evaluate thresholds and survival production
- Assess the point at which a colony is unable to recover
- Determine the impact inerts contained within pesticide formulations have on honey bees